Differential regulation of two forms of gonadotropin-releasing hormone messenger ribonucleic acid by gonadotropins in human immortalized ovarian surface epithelium and ovarian cancer cells

Jung-Hye Choi, Kyung-Chul Choi, Nelly Auersperg and Peter C K Leung

Department of Obstetrics and Gynecology, British Columbia Children's and Women's Hospital, Child and Family Research Institute, University of British Columbia, 2H-30, 4490 Oak St, Vancouver, British Columbia, Canada, V6H 3V5

(Requests for offprints should be addressed to P C K Leung; Email: peleung@interchange.ubc.ca)

Abstract

Although gonadotropin-releasing hormone (GnRH) has been shown to play a role as an autocrine/paracrine regulator of cell growth in ovarian surface epithelium and ovarian cancer, the factors which regulate the expression of GnRH and its receptor in these cells are not well characterized. In the present study, we employed real-time PCR to determine the potential regulatory effect of gonadotropins on the expression levels of GnRH I (the mammalian GnRH), GnRH II (a second form of GnRH) and their common receptor (GnRHR) in immortalized ovarian surface epithelial (IOSE-80 and IOSE-80PC) cells and ovarian cancer cell lines (A2780, BG-1, CaOV-3, OVCAR-3 and SKOV-3). The cells were treated with increasing concentrations (100 and 1000 ng/ml) of recombinant follicle-stimulating hormone (FSH) or luteinizing hormone (LH) for 24 h. Treatment with FSH or LH reduced GnRH II mRNA levels in both IOSE cell lines and in three out of five ovarian cancer cell lines (A2780, BG-1 and OVCAR-3). A significant decrease in GnRHR mRNA levels was observed in IOSE and ovarian cancer cells, except CaOV-3 cells, following treatment with FSH or LH. In contrast, treatment with either FSH or LH had no effect on GnRH I mRNA levels in these cells, suggesting that gonadotropins regulate the two forms of GnRH and its receptor differentially. In separate experiments, the effect of gonadotropins on the anti-proliferative action of GnRH I and GnRH II agonists in IOSE-80, OVCAR-3 and SKOV-3 cells was investigated. The cells were pretreated with FSH or LH (100 ng/ml) for 24 h after which they were treated with either GnRH I or GnRH II (100 ng/ml) for 2 days, and cell growth was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay. Pretreatment of the cells with FSH or LH significantly reversed the growth inhibitory effect of GnRH I and GnRH II agonists in these cell types. These results provide the first demonstration of a potential interaction between gonadotropins and the GnRH system in the growth regulation of normal ovarian surface epithelium and its neoplastic counterparts.

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Introduction

In addition to the classical form of mammalian gonadotropin-releasing hormone (GnRH I), a second form of GnRH (GnRH II) identical to chicken GnRH II has recently been found in the

brain of primates including humans (Lescheid *et al.* 1997). Besides the hypothalamus and pituitary gland, GnRH I, GnRH II and their mutual receptor (GnRHR) have also been shown to be expressed in extrapituitary tissues including the ovary (Dong *et al.* 1993, Kang *et al.* 2001c). In the ovary,

GnRH regulates the basal and gonadotropin-stimulated steroidogenesis in granulosa cells, and affects the expression of several genes related to folliculogenesis, ovulation and luteolysis (Peng *et al.* 1994, Kang *et al.* 2001b,c). Moreover, GnRH has been shown to be an autocrine regulator and has an anti-proliferative effect on gynecological cancers (Savino *et al.* 1992, Schally *et al.* 2001). In human ovarian surface epithelium and ovarian cancer, anti-proliferative and apoptosis inducing effects of GnRH have been demonstrated (Grundker & Emons 2003, Kang *et al.* 2003). Recent studies indicate that GnRH may modulate the growth of ovarian cancer cells by decreasing epidermal growth factor receptor (EGFR) expression and telomerase activity, and increasing the p53/p21 and Fas ligand-Fas system (Emons *et al.* 1993, Nagata & Golstein 1995, Ohta *et al.* 1998, Tang *et al.* 2002).

There is evidence that the expression of GnRH I, GnRH II and GnRHR is differentially regulated by its ligands, gonadal steroids (estrogen, progesterone and testosterone) and peptide hormones (activin, inhibin and gonadotropin) (Kang *et al.* 2001c, Khosravi & Leung 2003, Cheng & Leung 2005). In human ovarian surface epithelial (OSE) and granulosa luteal (GL) cells, treatment with GnRH I results in a biphasic response in its own ligand and receptor mRNA levels such that high concentrations decrease GnRH I and GnRHR mRNA levels whereas low concentrations increase the expression of both genes. In contrast, down-regulation of GnRH II and GnRHR mRNA levels was observed following treatment with GnRH II in GL cells (Kang *et al.* 2000, 2001c). Estradiol down-regulates GnRH I and GnRHR gene expression in GL cells and ovarian cancer (OVCAR-3) cells (Kang *et al.* 2001a, 2003). Gonadotropins have been shown to regulate the mRNA levels of GnRH I, GnRH II and GnRHR in the ovary (Peng *et al.* 1994, Olofsson *et al.* 1995, Kang *et al.* 2001c). In human GL cells, the expression of GnRH I and GnRH II is differentially regulated by follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) such that gonadotropins increase the mRNA levels of GnRH II but decrease those of GnRH I in a dose-dependent manner (Kang *et al.* 2001c). The expression of FSH receptor (FSHR) and luteinizing hormone receptor (LHR), and the growth-stimulating effect of their ligands in normal OSE and ovarian cancer cells has been demonstrated (Wimalasena *et al.* 1992, Kurbacher *et al.* 1995, Kobayashi *et al.* 1996, Mandai *et al.* 1997, Minegishi *et al.* 2000, Zheng *et al.* 2000, Ohtani *et al.* 2001, Parrott *et al.*

2001, Syed *et al.* 2001, Choi *et al.* 2002). However, the direct effect of gonadotropins on GnRH and GnRHR mRNA expression in human OSE and ovarian cancer cells remains to be elucidated. Considering that the two forms of GnRH may play an important role as autocrine/paracrine regulators in OSE and ovarian cancer, the present study was designed to investigate the role of gonadotropins in the regulation of GnRH I, GnRH II and GnRHR mRNA expression in human OSE and ovarian cancer cells. In addition, we also examined the ability of gonadotropins to modulate the growth-inhibitory effects of the two GnRHs.

Materials and methods

Cell culture and treatments

Human luteinizing hormone (LH) and recombinant FSH were provided by Dr AF Parlow (National Hormone and Pituitary Program, Harbor-University of California Los Angeles Medical Center, Torrance, CA, USA). GnRH I analog (D-Trp6-GnRH) and GnRH II analog (D-Arg6-Azagly10-GnRH II) were purchased from Bachem (Belmont, CA, USA). Non-tumorigenic SV40 Tag-immortalized OSE-derived cells (IOSE-80) were cultured as previously described (Choi *et al.* 2001b) in medium 199:MCDB 105 (1:1; Sigma-Aldrich Corp., St Louis, MO, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Ltd, Logan, UT, USA), 100 U/ml penicillin G and 100 mg/ml streptomycin (Life Technologies, Inc., Rockville, MD, USA) in a humidified atmosphere of 5% CO₂-95% air at 37 °C. At confluency, the cells were passaged with 0.06% trypsin (1:250)/0.01% EDTA in Mg²⁺/Ca²⁺-free Hank's Balanced Salt Solution (HBSS). The ovarian adenocarcinoma cell lines (A2780, BG-1, CaOV-3, OVCAR-3 and SKOV-3) were cultured in the above-mentioned culture conditions and used for the following experiments (Choi *et al.* 2001a). To investigate the regulation of GnRH I, GnRH II and GnRHR mRNA levels, cells were plated, cultured for 24 h, and then treated for an additional 24 h with FSH or LH (100 or 1000 ng/ml). Following treatment, the cells were lysed and immediately frozen at -70 °C until total RNA was extracted.

Real-time RT-PCR

Total RNA was prepared using TRIzol reagent (Invitrogen Canada, Burlington, ON, Canada), according to the manufacturer's instructions. Total

Table 1 Primers used for the detection of GnRHI, GnRHII, GnRHR, FSHR, LHR and GAPDH by SYBR Green RT-PCR

Gene		SYBR Green primer sequence (5' → 3')	% GC ^a	Tm ^b	Amplicon size (bp)
GnRHI	Forward	GCCTTAGAATGAAGCCAATTCAA	39	62	62
	Reverse	TCCACGCACGAAGTCAGTAGA	52	62	
GnRHII	Forward	TCTGTTCCCTCCAACCTTCTTC	48	63	64
	Reverse	AGGTCCATCCATCTTTCCTCA	46	62	
GnRHR	Forward	ACCGCTCCCTGGCTATCAC	63	63	60
	Reverse	ACTGTTCCGACTTTGCTGTTGCT	50	64	
FSHR	Forward	TTTCAAGAACAAGGATCCATTCC	39	61	68
	Reverse	CCTGGCCCTCAGCTTCTTAA	55	62	
LHR	Forward	TTCAATGGGACGACACTGACTT	46	62	64
	Reverse	TGTGCATCTTCTCCAGATGTACGT	46	63	
GAPDH	Forward	ATGGAAATCCCATCACCATCTT	41	62	57
	Reverse	CGCCCCACTTGATTTTGG	56	62	

^a% GC, guanine and cytosine percent of a primer sequence.

^bTm, melting temperature.

RNA (2.5 µg) was reverse transcribed into first-strand cDNA (Amersham Pharmacia Biotech, Oakville, ON, Canada) following the manufacturer's procedure. Briefly, the RNA solution was incubated at 65 °C for 10 min and then chilled on ice. Five microliters of the bulk first-strand cDNA reaction mix, 1 µl 200 mM dithiothreitol, 1 µl 0.2 µM Not I-d(T)18 primer and the heat-denatured RNA were mixed, and incubated at 37 °C for 1 h.

The primers used for SYBR Green real-time RT-PCR were designed using the Primer Express Software v 2.0 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and are listed in Table 1. These primers are specific for FSHR, LHR, GAPDH, GnRHI, GnRHII and GnRHR, as demonstrated using the BLAST program (<http://www.ncbi.nlm.nih.gov>), and were purchased from Invitrogen. To build a standard curve for each gene, cDNA fragments generated by RT-PCR were extracted from agarose gel bands and then used for 10-fold dilution. Real-time PCR was performed using the ABI prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems) equipped with a 96-well optical reaction plate. The reactions were set up with 12.5 µl SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems), 7.5 µl primer mixture (300 nM) and 5 µl cDNA template. Real-time PCR conditions were as follows: 52 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All real-time experiments were run in triplicate and a mean value was used for the determination of mRNA levels. Negative controls, containing water instead of sample cDNA, were

used in each real-time plate. The standard curve quantitation method (ABI PRISM 7700 Sequence Detection System User Bulletin #2) was used in this study, and the slope (S) of the trend line represents the PCR efficiency. Deviation from 100% efficiency was determined by the equation: PCR efficiency = $10^{(1/-S)} - 1$. The amount of transcript in each sample was calculated by interpolation using the following formula: (threshold cycle-y intercept)/S. The copy number of FSHR, LHR, GnRHI, GnRHII and GnRHR mRNAs in each cell line was normalized to the amount of GAPDH mRNA.

MTT assay

Cell viability was estimated using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay (Sigma-Aldrich Corp.). IOSE-80, OVCAR-3 and SKOV-3 cells were seeded in 96-well plates and incubated for 24 h. To examine the effect of FSH or LH on the growth-inhibitory effect of GnRHI and GnRHII agonists, IOSE-80, OVCAR-3 and SKOV-3 cells were pretreated with FSH or LH (100 ng/ml) for 24 h and then treated with GnRHI or GnRHII agonist for 2 days. On the day of collection, the cells were incubated at 37 °C with 50 µl MTT solution (2 mg/ml in phosphate-buffered saline (PBS)) for 4 h. The supernatants were removed and the cells were solubilized in DMSO (100 µl) for 30 min. The optical density at 570 nm was determined using a microplate spectrophotometer (Fisher Scientific Ltd, Ottawa, ON, Canada).

Data analysis

Data are shown as the mean $\pm$ S.D. of three individual experiments performed in triplicate, and are presented as the mean. For the MTT assay, values are expressed as the percentage of growth compared with control and are presented as the mean $\pm$ S.D. of three individual experiments performed in triplicate. Data were analyzed by one-way ANOVA followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

Results

Validation of real-time RT-PCT for FSHR, LHR, GAPDH, GnRH I, GnRH II and GnRHR

To examine whether the RT-PCR conditions produced primer-dimers and multiple amplicons, dissociation curve analysis and agarose gel electrophoresis were performed. Typical standard curves for the real-time amplification of FSHR, LHR, GnRH I, GnRH II, GnRHR and GAPDH were constructed (data not shown). Agarose gel electrophoresis of the amplicons yielded a single band (data not shown). The standard curve was log-linear for seven orders of magnitude (from 10^2 to 10^8 copies) and the coefficient of regression (r^2) was >0.98 . A dissociation curve analysis of FSHR, LHR, GnRH I, GnRH II and GnRHR amplicon resulted in a single peak (data not shown). The identity of the amplicons was confirmed to be FSHR, LHR, GnRH I, GnRH II, GnRHR and GAPDH by sequencing analysis.

Expression of FSHR and LHR in IOSE and ovarian cancer cells

In a recent study, we demonstrated the expression of FSHR protein in IOSE cells and ovarian cancer cells using Western blot. Low levels of FSHR protein were shown in IOSE-80PC (an IOSE-80 line derived post-crisis) and SKOV-3 (a potential invasive line) cells, whereas FSHR was highly expressed in OVCAR-3 cells (up to sevenfold higher) (Choi *et al.* 2004). In the present study, we evaluated the mRNA levels of FSHR and LHR among two IOSE cell lines (IOSE-80 and IOSE-80PC) and five ovarian cancer cell lines (A2780, BG-1, CaOV-3, OVCAR-3 and SKOV-3) using real-time RT-PCR (Fig. 1). We found that all seven cell lines expressed FSHR and LHR mRNA more than 0.0001 copy (ratio to GAPDH). The expression pattern of FSHR mRNA among IOSE-80, OVCAR-3 and SKOV-3 cells was similar to that of FSHR protein

demonstrated in our previous study (Choi *et al.* 2004). No significant difference in either FSHR or LHR mRNA levels was observed between IOSE cells and ovarian cancer cells.

Effect of FSH and LH on GnRH I, GnRH II and GnRHR

To investigate whether GnRH I, GnRH II and GnRHR expression is regulated by FSH or LH in IOSE and ovarian cancer cell lines, the expression levels of GnRH I, GnRH II and GnRHR mRNA were examined following treatment of these cells with FSH or LH. The concentration of FSH and LH (100 and 1000 ng/ml) was selected from previous results which showed that these doses caused functional changes in OSE or ovarian cancers (Choi *et al.* 2002, Pon *et al.* 2005). In all the cells examined including IOSE and ovarian cancer cells, no significant change in GnRH I mRNA levels was observed following treatment with gonadotropins (Fig. 2). However, treatment with gonadotropins induced a significant decrease ($\sim 60\%$) in GnRH II mRNA levels in two IOSE cells and in three ovarian cancer cells (A2780, BG-1, OVCAR-3), but not in CaOV-3 and SKOV-3 cells (Fig. 3). In addition, treatment with FSH or LH for 24 h resulted in a significant down-regulation of GnRHR mRNA in all the cell lines examined except CaOV-3 cells; however this effect was greater in IOSE cells than in ovarian cancer cells (Fig. 4). For example, in IOSE-80 cells, both FSH and LH decreased GnRHR mRNA in a dose-dependent manner with maximal 60% and 80% decreases at 1000 ng/ml respectively. In contrast, maximal 40% and 60% decreases were observed at 1000 ng/ml FSH and LH in OVCAR-3 cells. These data suggest that gonadotropins may differentially regulate the expression of both forms of GnRH and their receptor in ovarian surface epithelium and its neoplastic counterpart.

Effect of FSH and LH treatment on the growth inhibitory effect of GnRH I and GnRH II in IOSE-80 and OVCAR-3 cells

It has been demonstrated that agonistic analogs of both GnRH I and GnRH II inhibit the growth of IOSE, OVCAR-3 and SKOV-3 cells (Kang *et al.* 2003). As gonadotropins decreased the mRNA expression of GnRH II and GnRHR, we used the MTT assay to further examine whether gonadotropins modulate the growth inhibition of IOSE-80, OVCAR-3 and SKOV-3 cells by GnRH agonists.

Figure 1 Expression of (A) FSHR and (B) LHR in IOSE and ovarian cancer cells. First-strand cDNA from IOSE-80, IOSE-80PC, A2780, BG-1, CaOV-3, OVCAR-3 and SKOV-3 cells was amplified using two sets of PCR primers shown in Table 1. The expression levels of FSHR and LHR mRNA were normalized against the GAPDH mRNA level. Data are derived from three experiments and are presented as the mean $\pm$ s.d.

Figure 2 Effect of FSH and LH on GnRH mRNA in IOSE and ovarian cancer cells. The cells were plated and cultured for 24 h. The cells are then treated with FSH (100 and 1000 ng/ml) or LH (100 and 1000 ng/ml) for 24 h. Control cultures were treated with vehicle. Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of GnRH mRNA were measured by real-time RT-PCR. Data are presented as the mean $\pm$ s.d. of three experiments.

Figure 3 Effect of FSH and LH on GnRHII mRNA in IOSE and ovarian cancer cells. The cells were plated and cultured for 24 h. The cells are then treated with FSH (100 and 1000 ng/ml) or LH (100 and 1000 ng/ml) for 24 h. Control cultures were treated with vehicle. Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of GnRHII mRNA were measured by real-time RT-PCR. Data are presented as the mean $\pm$ S.D. of three experiments. a, $P < 0.05$ compared with the control of each cell line.

The cells were pretreated with gonadotropin (100 ng/ml) or vehicle for 24 h and then treated with GnRH I or GnRH II agonist (10^7 M), in the presence or absence of gonadotropin, for 48 h. The dose and time of GnRH treatment (10^7 M for 48 h) were selected based on a previous study (Kang *et al.* 2000). In agreement with our previous findings (Choi *et al.* 2002), treatment with FSH alone resulted in a significant increase in growth of both

IOSE-80 and OVCAR-3 cells, but not SKOV-3 cells. In contrast, treatment with LH alone showed a mitogenic effect only in OVCAR-3 cells. GnRH I and GnRH II agonists significantly inhibited the growth of all three cell lines examined. Pretreatment with gonadotropins for 24 h completely reversed the growth-inhibitory effect of GnRH I and GnRH II agonists in IOSE-80 (Fig. 5A), OVCAR-3 (Fig. 5B) and SKOV-3 cells (Fig. 5C).

Figure 4 Effect of FSH and LH on GnRHR mRNA in IOSE and ovarian cancer cells. The cells were plated and cultured for 24 h. The cells are then treated with FSH (100 and 1000 ng/ml) or LH (100 and 1000 ng/ml) for 24 h. Control cultures were treated with vehicle. Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of GnRHR mRNA were measured by real-time RT-PCR. Data are presented as the mean $\pm$ S.D. of three experiments. a, $P < 0.05$ compared with the control of each cell line.

Figure 5 Effect of FSH or LH treatment on the growth inhibitory effect of GnRH I and GnRH II in IOSE-80 cells (A), OVCAR-3 cells (B) and SKOV-3 cells (C). The cells were seeded on 96-well plates and incubated for 24 h. Cells were pretreated with FSH or LH (100 ng/ml) for 24 h and then treated with an analog of GnRH I (D-Trp6-GnRH) or GnRH II (D-Arg6-Azagly10-GnRH II) for 2 days. Cell growth was measured using the MTT assay as described in the Materials and methods section. Data are presented as the mean $\pm$ S.D. of three experiments. a, $P < 0.05$ compared with control; b, $P < 0.05$ compared with GnRH I or GnRH II agonist.

Discussion

Ovarian cancers, mainly derived from the ovarian surface epithelium, are the most lethal gynecological malignancy and are the fifth leading cause for all cancer deaths in women (Auersperg *et al.* 2001). There is increasing evidence suggesting the positive or negative effect of reproductive hormones including GnRH and gonadotropins on ovarian cancer initiation and progression (Riman *et al.* 1998, Risch 1998, Brekelmans 2003). It is of interest that ovarian cancer is more common in conditions with elevated gonadotropins such as post-menopausal women (Holschneider & Berek 2000, Brekelmans 2003). Reduced risk of ovarian cancer is associated with multiple pregnancies, breast feeding, oral contraceptives, and estrogen replacement therapy which are associated with lower levels and reduced exposure to gonadotropins (Daly & Obrams 1998, Gnagy *et al.* 2000, La Vecchia 2001). Moreover, it has been demonstrated that gonadotropin levels of ovarian cyst fluid significantly increase in patients with ovarian cancer as compared with patients with functional and benign ovarian cysts (Halperin *et al.* 2003, Chudecka-Glaz *et al.* 2004).

The expression of FSHR and LHR in normal OSE and ovarian cancer cells has been demonstrated (Kobayashi *et al.* 1996, Mandai *et al.* 1997, Minegishi *et al.* 2000, Zheng *et al.* 2000, Parrott *et al.* 2001). However, the role of FSH and LH in normal OSE and ovarian epithelial cancer is not well characterized. Although a gonadotropin theory that they may be involved in the development of epithelial ovarian cancer is still controversial to date (Wimalasena

et al. 1991, Venn *et al.* 1995, Ivarsson *et al.* 2001, Tourgeman *et al.* 2002), it is assumed that FSH and LH/hCG stimulate the growth of normal, immortalized OSE and some ovarian cancer cells in a dose- and time-dependent manner *in vitro* (Wimalasena *et al.* 1992, Kurbacher *et al.* 1995, Ohtani *et al.* 2001, Parrott *et al.* 2001, Syed *et al.* 2001, Choi *et al.* 2002). In the present study, we examined the alteration in GnRH I, GnRH II and GnRHR mRNA expression in human OSE and ovarian cancer cells by FSH and LH at concentrations that are associated with the relatively high levels of gonadotropins in post-menopausal women. After the menopause there is a 10- to 20-fold increase in gonadotropins compared with basal levels in the normal reproductive cycle. We also examined the ability of gonadotropins to modulate the growth-inhibitory effects of two forms of GnRHs.

GnRH I and its receptor are expressed in 80% of human OSE cells and ovarian cancer cell lines (Emons *et al.* 1993, Miyazaki *et al.* 1997), suggesting that this decapeptide may be an autocrine and/or paracrine regulator of the OSE and may play a role in the pathophysiology of ovarian cancer (Savino *et al.* 1992, Schally 1999, Schally *et al.* 2001, Grundker & Emons 2003, Kang *et al.* 2003). In the present study, we found that four out of five ovarian cancer cell lines (A2780, BG-1, CaOV-3 and OVCAR-3) highly expressed basal GnRHR compared with IOSE cells. This is consistent with a recent report that primary ovarian cancer cells expressed higher levels of GnRHR compared with normal ovarian tissues using RT-PCR, immunohistochemistry and Western blot assay. For instance,

the mean values of fold-increase of GnRHR mRNA level in stage I–IV ovarian cancer were 2.24, 2.58, 3.10 and 3.20 as compared with normal ovarian tissues, and overall 70% (21/30) of ovarian cancer tissues had increased GnRHR mRNA levels.

As shown in Figs 2 and 3, SKOV-3 cells with potential invasiveness in our cell culture system highly expressed both GnRH I and GnRH II compared with IOSE cells, whereas other ovarian cancer cells including A2780, BG-1 and OVCAR-3 showed modest increased levels of GnRH II. Whether the expression of GnRHs as well as their receptor could be used to characterize the histological type, stage or grade of human ovarian epithelial cancer remains to be determined. In view of the recent observation that the GnRH/GnRHR system can be modulated by gonadotropins in hypothalamic GT1-7 neuron and human granulosa luteal cells (Lei & Rao 1994, Kang *et al.* 2001c), we hypothesized that gonadotropins would interact with the GnRH/GnRHR system to regulate cell growth in OSE and ovarian cancer cells. This hypothesis is supported by the results of the present study that GnRH II and/or GnRHR mRNA levels are down-regulated by gonadotropin in IOSE cells and ovarian cancer cells. It cannot be explained why the regulatory effect of gonadotropin on the GnRH/GnRHR system is relatively weak in SKOV-3 cells, such that FSH and LH can modulate only GnRHR (with a maximum 40% decrease), and is absent in CaOV-3 cells. Our results suggest that the two forms of GnRH are differentially regulated under various physiological conditions. Indeed, in the brain of the female European silver eel, steroids induce an increase in mammalian (m) GnRH and a decrease in chicken (c) GnRH II (Montero *et al.* 1995). In the chicken, only the level of mGnRH in the hypothalamus was modified by castration (Sun *et al.* 2001). In the goldfish, the ratio between salmon GnRH and cGnRH II changes with sexual maturation (Rosenblum *et al.* 1994).

Treatment of human GL cells with FSH and hCG resulted in a marked increase in GnRH II mRNA levels but decreased those of GnRH I (Kang *et al.* 2001c). This differential regulation of two forms of GnRH by various factors including gonadotropins in a range of cell types suggests the distinct spatial expression and function of these peptides. Furthermore, considering the growth inhibitory effect of GnRH, decreased expression of GnRH II or the GnRH II/GnRH I ratio may play a critical role in the development of ovarian cancer by regulating

proliferation. The reason why gonadotropins differentially regulate the transcription of GnRH I and GnRH II genes is not clear. It is possible that two distinct transcription mechanisms exist for each GnRH in the ovary and that gonadotropins regulate only the GnRH II-related mechanism. Further study is required to elucidate the physiological relevance and the mechanism of the differential regulation of GnRH I and GnRH II by gonadotropins.

Previously, we and others demonstrated that treatment with GnRH I and GnRH II inhibits the proliferation of IOSE and ovarian cancer cells as determined by thymidine incorporation (Grundker & Emons 2003, Kang *et al.* 2003). In this study, we further explored the possibility that gonadotropins may antagonize the growth inhibitory effect of GnRH I and GnRH II in IOSE, OVCAR-3 and SKOV-3 cells by regulating the expression levels of GnRH ligands and their receptor. The growth-inhibitory effect of GnRH I and GnRH II was substantiated further in the present study using the MTT assay. A decrease in cell number of approximately 20% was observed in response to GnRH I or GnRH II in IOSE-80, OVCAR-3 and SKOV-3 cells, and this effect was reversed by pretreatment with FSH or LH. These results indicate that gonadotropins may function as growth regulators in IOSE-80, OVCAR-3 and SKOV-3 cells. The mechanism of action of gonadotropins in this regard is not clear. We cannot rule out the possibility that the mitogenic activity of gonadotropins may override the growth inhibitory activity of GnRH analogs independent of down-regulation of GnRH II and GnRHR in these cells. As shown in Fig. 5C, pretreatment with gonadotropin, which is not a mitogen in SKOV-3 cells, reversed GnRH analog-inhibited cell growth. This result suggests that the mechanism of action of gonadotropins may include, at least in part, a reduction in GnRH II and GnRHR mRNA levels. In addition, the effects of FSH and LH in these cells may also be indirect via other growth factors. For instance, combined treatment with hCG and estradiol may regulate the growth of epithelial ovarian cancer cells through the insulin-like growth factor-I pathway (Wimalasena *et al.* 1993). Likewise, FSH and hCG stimulate steady state mRNA levels of keratinocyte growth factor, hepatocyte growth factor and kit ligand in bovine OSE cells (Parrot 2001). Recently, we have demonstrated that treatment of immortalized OSE and OVCAR-3 cells with FSH and LH significantly increased EGFR mRNA and protein (Choi *et al.* 2005).

In summary, a significant decrease in GnRH II and GnRHR mRNA levels was observed in IOSE and ovarian cancer cells following treatment with FSH or LH. In contrast, treatment with either FSH or LH had no effect on GnRHI mRNA levels in the cell lines employed, suggesting that gonadotropins regulate these two forms of GnRH and its receptor differentially. Pretreatment of the cells with FSH or LH significantly reversed the growth inhibitory effect of GnRHI and GnRHII agonists in IOSE-80, OVCAR-3 and SKOV-3 cells. Taken together, these results suggest that FSH and LH may interact with the GnRH system to control the growth of ovarian surface epithelial cells and their neoplastic counterparts.

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